Short Telomeres Limit Tumor Progression In Vivo by Inducing Senescence

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SUMMARY

Telomere maintenance is critical for cancer progression. To examine mechanisms of tumor suppression induced by short telomeres, we crossed mice deficient for the RNA component of telomerase, mTR−/−, with Eμ-myc transgenic mice, an established model of Burkitt’s lymphoma. Short telomeres suppressed tumor formation in Eμ-myc transgenic animals. Expression of Bcl2 blocked apoptosis in tumor cells, but surprisingly, mice with short telomeres were still resistant to tumor formation. Staining for markers of cellular senescence showed that pretumor cells induced senescence in response to short telomeres. Loss of p53 abrogated the short telomere response. This study provides in vivo evidence for the existence of a p53-mediated senescence mechanism in response to short telomeres that suppresses tumorigenesis.

INTRODUCTION

Burkitt’s lymphoma is a highly aggressive, rapidly growing, and lethal cancer. Tumors that arise are uniformly associated with translocations that activate the c-myc oncogene. A transgenic mouse model of Burkitt’s lymphoma in which c-myc is expressed in B cells (Adams et al., 1985) provides an excellent model to understand the biology of lymphoma and to test mechanisms that may limit tumor growth. Understanding the pathways that interrupt tumor growth will allow the development of specific therapies for lymphoma.

Cells contain two major intrinsic pathways of tumor suppression, apoptosis and senescence, that can be activated by multiple stimuli (Lowe et al., 2004). Telomerase inhibition was proposed as a potential cancer therapy when telomere shortening was first described in human cells (Harley et al., 1990). Telomerase is critical for telomere length maintenance; when telomerase is absent or blocked, progressive telomere shortening occurs with each cell division. In primary cultures of human cells, telomere shortening results in dysfunctional telomeres, which trigger a DNA damage response that ultimately leads to an irreversible state of cellular senescence (Bodnar et al., 1998; d’Adda di Fagagna et al., 2003). In mouse cells, short telomeres also initiate a DNA damage response (Hao et al., 2004); however, in response, these cells do not enter senescence (Blasco et al., 1997; Parrinello et al., 2003). In vivo short telomeres induce apoptosis in multiple highly proliferative tissues, including testis germ cells and lymphocytes (Hemann et al., 2001a; Lee et al., 1998). Senescent cells were detected in a subset of hepatocytes that were induced to proliferate via partial hepatectomy in mice with dysfunctional telomeres (Lechel et al., 2005; Satyanarayana et al., 2003). However, senescence was not observed in quiescent hepatocytes in animals with acute telomere dysfunction due to telomere uncapping (Denchi et al., 2006). Strong evidence has accumulated that short telomeres indeed limit tumor growth. Crosses of mTR−/− mice to tumor-prone models demonstrate that the short

SIGNIFICANCE

Telomerase inhibition has potential therapeutic benefits for cancer treatment. Experimental systems indicate that short telomeres impair tumor formation by inducing apoptosis. Therefore, blocking apoptosis was expected to block the tumor-suppressive effect of short telomeres. However, we found that, despite efficient inhibition of apoptosis, short telomeres retained potent tumor-suppressive signaling in Eμ-myc-induced lymphomas. This response required p53 and showed hallmarks of cellular senescence. Thus, telomerase inhibition can engage multiple p53-dependent tumor suppressor pathways and may have therapeutic potential for tumors that have lost specific p53 effector functions but retain wild-type p53.
telomere response significantly limits tumor formation (Gonzalez-Suarez et al., 2000; Greenberg et al., 1999; Qi et al., 2003, 2005; Rudolph et al., 2001; Wong et al., 2003). In these experiments, decreased tumor formation correlated with an increase in DNA damage, chromosome instability, and apoptosis. However, senescence was not reported in these studies. Taken together, these observations raise the question whether short telomere-induced senescence may act as a tumor suppressor mechanism.

The p53 tumor suppressor protein is the major mediator of the DNA damage response, apoptosis, and senescence. Consistent with this role, p53 deficiency abrogates many of the cellular responses to short telomeres (Chin et al., 1999). In addition, short telomeres stimulated the formation of tumors in p53-deficient mice and caused the occurrence of tumor types that are not normally associated with this tumor-prone mouse model (Artandi et al., 2000). The loss of p53 confers multiple advantages to uncontrolled proliferation, including loss of the DNA damage checkpoint, genome stability, senescence, and apoptosis (Vogelstein et al., 2000). It is therefore unclear which p53 effector functions are important for tumor suppression in response to short telomeres.

In the work presented here, we demonstrate that short telomeres suppress tumorigenesis in a mouse model of Burkitt’s lymphoma and examine the requirement of apoptosis in mediating this response. We found that, surprisingly, inhibition of apoptosis did not abrogate the reduction in lymphoma formation in response to short telomeres. After long latency, microlymphomas that did appear displayed multiple markers of senescence. Genetic evidence showed that p53 was absolutely required for tumor suppression. We conclude that short telomeres activate a p53-dependent cellular senescence pathway that limits tumor formation in vivo.

RESULTS

The Eμ-MyC transgenic mouse is an established model of Burkitt’s lymphoma. Overexpression of the Myc oncogene in B cells leads to B cell lymphoma with a median onset of 4–6 months (Adams et al., 1985). To assess the impact of short telomeres on lymphomagenesis, we generated Eμ-MyC transgenic mice that were deficient for the RNA component of telomerase (Blasco et al., 1997) (Eμ-MyC;mTR−/−). To obtain Eμ-MyC transgenic mice
with short telomeres, we intercrossed $E_{\mu}$-myc;mTR$^{-/-}$ mice for six successive generations in the absence of telomerase. This breeding strategy allows for the comparison of $E_{\mu}$-myc transgenic mice that are wild-type for telomerase (Myc;mTR$^{+/+}$), those that lack telomerase but have long telomeres (Myc;mTR$^{-/-}$ G1), and those that lack telomerase but have short telomeres (Myc;mTR$^{-/-}$ G5/6).

To determine the effect of short telomeres on tumor formation, we monitored cohorts of Myc;mTR$^{+/+}$, Myc;mTR$^{-/-}$ G1, and Myc;mTR$^{-/-}$ G5/6 mice for lymphoma onset. Consistent with previous studies (Adams et al., 1985), Myc;mTR$^{+/+}$ and Myc;mTR$^{-/-}$ G1 animals developed fully penetrant B cell lymphoma, and the latency of lymphoma onset was indistinguishable between the two cohorts (Figure 1A). In contrast, only 7 of 25 Myc;mTR$^{-/-}$ G5/6 animals developed lymphoma over the course of the study ($p < 10^{-4}$, Figure 1A). As expected, telomere length was significantly shorter in Myc;mTR$^{-/-}$ G5/6 tumors compared to Myc;mTR$^{+/+}$ and Myc;mTR$^{-/-}$ G1 tumors (Figure 1B). Further, these tumors displayed a high frequency of chromosome end-to-end fusions and nonreciprocal translocations (Figures 1C and 1D). In contrast, Myc;mTR$^{+/+}$ and Myc;mTR$^{-/-}$ G1 tumors showed no nonreciprocal translocations and only one independent, but clonal, end-to-end chromosome fusion. These observations indicate that short telomeres induce extensive genomic instability and are a potent inhibitor of tumorigenesis in $E_{\mu}$-myc transgenic mice.

Previous studies in telomerase-deficient mice have suggested that the apoptotic pathway is critical for short telomere-mediated tumor suppression (Gonzalez-Suarez et al., 2000; Greenberg et al., 1999; Qi et al., 2003, 2005; Rudolph et al., 2001; Wong et al., 2003). To directly test the requirement of apoptosis for short telomere-mediated tumor suppression, we disabled apoptotic signaling by expressing the Bcl2 oncogene using an adoptive transfer protocol (Figure 2A). We cultured bone marrow, enriched for hematopoietic stem cells, from young, nonlymphocytic Myc;mTR$^{+/+}$, Myc;mTR$^{-/-}$ G1, and Myc;mTR$^{-/-}$ G5/6 animals. Whole bone marrow was infected with a murine stem cell virus prior to transplantation into lethally irradiated syngeneic recipient animals. Iconography reproduced by kind permission of New Science Press from DeFranco et al., 2007.

Adoptive transfer of Bcl2 expressing $E_{\mu}$-myc bone marrow into lethally irradiated syngeneic recipient animals results in rapid lymphoma onset approximately 6 weeks posttransplant (Schmitt et al., 2002). Disabling apoptosis results in a rapid onset of tumors during $E_{\mu}$-myc lymphomagenesis (Schmitt et al., 2002). As expected, transplantation of Bcl2-positive HSCs derived from Myc;mTR$^{+/+}$ and Myc;mTR$^{-/-}$ G1 animals resulted in rapid tumor onset in all recipient animals in both genotypes (8 of 8 Bcl2;Myc;mTR$^{+/+}$ and 7 of 8 Bcl2;Myc;mTR$^{-/-}$ G1 mice were lymphoma bearing by 42 days posttransplant Figure 2B). These animals harbored highly aggressive tumors displaying complete effacement of the cervical lymph nodes and infiltration of tumor cells into adjacent salivary gland. Surprisingly, animals transplanted with Bcl2-positive Myc;mTR$^{-/-}$ G5/6 bone marrow failed to develop palpable tumors for more than 100 days posttransplant (0 of 3 Myc;mTR$^{-/-}$ G5 [129 days] and 0 of 5 Myc;mTR$^{-/-}$ G6 [100 days]; Figure 2B). Histological evaluation of lymphatic tissues from these animals showed small encapsulated tumor masses in the cervical lymph nodes with little infiltration into salivary gland (Figure 5B). To confirm that Bcl2 was blocking apoptosis in the tumor cells in these animals, we used a terminal UTP nick end labeling (TUNEL) protocol to examine the extent of apoptosis in lymphomas from Bcl2-expressing mice. Apoptotic levels were significantly reduced in Bcl2-expressing
lymphomas compared to spontaneously arising Eμ-myc lymphomas that do not express Bcl2 (Figure 2C). Importantly, the amount of apoptotic cells was similar in all Bcl2-positive lymphomas tested (Student’s t test; p = 0.6 for Myc;TR+/+ to Myc;TR−/− mice, n = 9). These data indicate that short telomeres effectively abolished tumor progression even when apoptosis was blocked.

Decreased tumor formation in the absence of apoptosis suggested that another mechanism is limiting tumor growth. p53 regulates multiple pathways that act to suppress tumor formation, including apoptosis and senescence (Lowe et al., 2004; Vogelstein et al., 2000). To genetically test whether p53 is required for the short telomere-induced suppression of lymphoma, we crossed mice doubly heterozygous for mTR and p53 (mTR+/−; p53+/−) to Myc;TR−/− G6 mice (Figure 3A). This crosses in intergenerational G7 (iG7) littersmates that all have half-long and half-short telomeres and are either telomerase positive (mTR−/−) or telomerase negative (mTR−/+).

The mTR−/+ iG7 mice have short telomeres that mimic mTR−/− G6 mice (Feldser et al., 2006; Hemann et al., 2001b; Qi et al., 2003). Eμ-myc transgenic animals that are heterozygous for p53 (p53+/−) invariably develop Myc;p53−/− tumors at ∼40 days. Short telomeres promote chromosomal abnormalities in p53-deficient lymphomas.

Figure 3. Short Telomeres Block Tumor Formation in a p53-Dependent Manner

(A) Intergenerational breeding scheme. Vertical bars represent telomere lengths.

(B) Kaplan-Meier survival analysis of Myc;TR−/−;p53+/− (black solid line, n = 11), Myc;TR−/−;p53+/− (red solid line, n = 19), Myc;TR−/−;p53+/− (black dashed line, n = 11), and Myc;TR−/−;p53+/− (red solid line, n = 8) cohorts. Myc;p53−/− animals invariably develop Myc;p53−/− tumors at ∼40 days. Short telomeres promote chromosomal abnormalities in p53-deficient lymphomas.

(C) Q-FISH analysis of metaphase spreads from Myc;TR−/−;p53+/− (top) and Myc;TR−/−;p53+/− (bottom) lymphomas. Ten metaphases each from three separate lymphomas are represented for each genotype.

(D) Quantitation of chromosome abnormalities. The number of chromosome end-to-end fusions (red bars) and nonreciprocal translocations (blue bars) per metaphase are shown (ten metaphases per tumor, n = 3). Error bars indicate standard error.
In addition to these genetic data, several lines of evidence suggest that loss of p53 is critical for tumor development in the presence of short telomeres. First, tumors that are derived from Myc;mTR−/−G5/6 animals (Figure 1) are unable to elicit a G1 checkpoint in response to γ-IR, and these tumors also do not initiate apoptosis after γ-IR treatment (Figures 4A, 4B, and 4D). Second, immunoblot analysis showed that, in these tumors, p53 was either undetectable regardless of γ-IR treatment or grossly overexpressed and uninducible (Figure 4C). Finally, p19ARF was overexpressed in all tumors from Myc G5/6 animals (6 out of 6 lymphomas tested, \( \chi^2 \) test \( p = 0.01 \) compared to Myc;mTR−/−). These observations are consistent with p53 inactivation in the tumors, either by deletion or by point mutation, which impairs p53 degradation (Eischen et al., 1999). This implies that, for tumors to grow in the presence of short telomeres, the p53 pathway must be inactivated.

The requirement for p53, but not apoptosis, implies that p53-mediated cellular senescence may be responsible for tumor suppression. To determine whether short telomeres could be blocking tumor progression by initiating senescence, we analyzed microlymphomas from Bcl2;Myc; mTR−/−G5/6 animals (Figure 2) for proliferation defects and the presence of senescence markers. Compared to Bcl2;Myc;mTR−/− sections, Bcl2;Myc;mTR−/−G5/6 sections had significantly fewer mitotic figures per high-power field (Figure 5A; \( p < 0.0001 \)), indicating that short telomeres negatively affect cellular proliferation.

Next, Bcl2;Myc lymphomas were analyzed for the presence of markers that are specifically upregulated in senescent tissues (Collado and Serrano, 2006). Bcl2-expressing Myc;mTR−/−G5/6 lymphomas displayed both p16INK4a and p15INK4b immunohistochemical staining. In contrast, Bcl2-expressing Myc;mTR−/− lymphomas did not stain for any of these markers (Figure 5B). These staining patterns indicate that Bcl2-expressing Myc;mTR−/−G5/6 microlymphomas were indeed senescent. Taken together with the failure of these lymphomas to progress, these data indicate that
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DISCUSSION

Understanding cellular mechanisms that may limit tumor growth could provide new therapeutic approaches to cancer therapy. Telomere shortening, through the inhibition of telomerase, has been proposed as a potential cancer treatment. Here we show that short telomeres can block lymphoma induction in a mouse model of Burkitt’s lymphoma. Short telomeres have been shown to reduce tumor formation in several other mouse tumor models (Gonzalez-Suarez et al., 2000; Greenberg et al., 1999; Qi et al., 2003, 2005; Rudolph et al., 2001; Wong et al., 2003); however, in all of those systems, apoptosis was thought to be responsible for the decreased incidence of tumor formation. We found that blocking apoptosis still resulted in tumor suppression. p53 was required for the tumor reduction due to short telomeres, and direct evidence indicated that a p53-mediated senescence program was activated in the small tumors that did form. These results indicate that short telomeres can mediate a p53-dependent senescence program that limits tumor formation in vivo.

Telomere-mediated senescence in tumors may not have been detected in previous studies because it likely is initiated only in a few cells. Recently, cellular senescence induced by oncogene expression has been established as a tumor suppressor mechanism in vivo (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Michaloglou et al., 2005). Activation of cellular senescence in these instances occurs in all cells in the initiating tumor mass, thus facilitating detection of senescence markers. In contrast, senescence induced by short telomeres may occur initially in only a few cells in which telomeres become critically short. This may impede the detection of short-telomere-mediated senescence during tumor formation.

Short telomeres can engage both the apoptosis and the senescence pathways, p53 is necessary to carry out both apoptotic and senescence pathways (Figure 6). The requirement of the loss of p53 for the growth of the Eμ-myc lymphomas with short telomeres suggests both apoptosis and senescence play roles in limiting tumor growth. If only one tumor suppressor pathway, apoptosis or senescence, were necessary for short-telomere-mediated tumor suppression, we would have expected mutations in other components of these pathways to also allow tumor formation in these mice. For example, if disruption of apoptosis were the only p53 effector function required for tumor formation in the presence of short telomeres, we would have expected overexpression of Bcl2 to confer a selective growth advantage regardless of telomere length. Consistent with this, we speculate that loss of p16 pathway components in tumors that express Bcl2 would be resistant to the tumor-suppressive effects of short telomeres (Figure 6). Tumor suppressor programs induced by p53 are context dependent. Restoration of p53 function in p53 null tumors induces tumor regression in multiple tumor types (Martins et al., 2006; Ventura et al., 2007; Xue et al., 2007). However, p53 restoration induces apoptosis in lymphomas (Martins et al., 2006; Ventura et al., 2007) and senescence in sarcomas (Ventura et al., 2007) and liver carcinomas (Xue et al., 2007). It is unclear whether p53-induced senescence or apoptosis correlates with cell type, oncogenic lesion, or both. We show here that, in a tumor type that is predisposed to p53-mediated apoptosis, short telomeres can effectively redirect the p53 tumor suppressor response to induce senescence. It would be interesting if short telomeres could, likewise, alter p53-mediated senescence programs to induce apoptosis in tumor types predisposed to undergo senescence.
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**EXPERIMENTAL PROCEDURES**

**Lymphoma Monitoring and Analysis**
The Institutional Animal Care and Use Committee at the Johns Hopkins University approved all animal procedures. Eμ-myc transgenic animals were inspected twice weekly for lymph node enlargement by palpation and for overall signs of distress. Animals were considered lymphomic when tumor mass was easily palpated no more than 1 cm in diameter. Lymphoma-bearing animals were euthanized, and tumor masses were dissociated through nylon mesh into single-cell suspensions and cultured on mitomycin C-treated 3T3 feeder layers in B cell media (45% DMEM [Invitrogen], 45% IMDM [Invitrogen], 10% FBS [Hyclone], Pen/Strep/Glutamine [Invitrogen]).

**Chromosome Analysis**
Metaphase spreads were generated by arresting exponentially growing cultures with 0.5 μg/ml KaryoMax (Gibco) 2 hr before hypotonic swelling in 0.075 M KCl and fixation in 3:1 methanol:acetic acid. Fixed cells were dropped onto glass slides over a steaming water bath. Telomere length was determined by hybridization of metaphases with Cy3 (CCCTAA), PNA probes (PE Biosystems) as described (Lansdorp et al., 1996). Spectral karyotyping was performed on metaphases as described (Liyanage et al., 1998). Images were obtained using IP-Lab software on a Zeiss Axioscope microscope.

**Immunoblot, Apoptosis, and Cell-Cycle Analysis**
Exponentially growing lymphoma cells (2 × 10^6) were grown in 6-well plates. Cells were exposed to 0 or 5 Gy γ-IR from a cesium source (Gamma Cell) prior to analyses. For immunoblot analysis, cells were lysed in RIPA buffer containing protease inhibitors (Roche), denatured in SDS loading buffer, separated on a 4%–12% gradient Tris-acetate gel (Novex), transferred to PVDF (MilliPore), and probed with anti-p53 (CM5 Novocastra), anti-Arf (Ab80 Abcam), and anti-actin (Sigma). For apoptosis analysis, cells were harvested, washed in PBS, and then stained with an Annexin-V FLUOS staining kit (Roche) per the manufacturer’s instructions. For BrdU incorporation, BrdU was added to culture medium 1 hr postirradiation, and analysis was performed 6 hr after γ-IR. Cells were harvested and analyzed using an in situ cell proliferation kit (Roche) per the manufacturer’s instructions.

**Bone Marrow Transplantation Protocol and Retrovirus Production**
Donor animals (6- to 10-week-old on C57BL/6J background) were primed with 5-FU (150 mg/kg) 4 days prior to bone marrow harvest. Marrow was harvested from tibia and femur by flushing with Hank’s balanced salt solution with a 23-gauge needle. After red cell lysis, cells were cultured in IMDM with 18% FBS (Hyclone) 4% WEHI-3 conditioned media, 10 ng/ml IL-3, 10 ng/ml IL-6, and 100 ng/ml mSCF (Fitzgerald). Ecotropic retrovirus was produced using Phoenix cells (G. Noll, Stanford) by transfecting 12 μg MSCV:Bcl2 (Schmitt et al., 2002) into 2 × 10^6 cells with 20 μl Lipofectamine 2000 (Invitrogen) overnight. Virus was collected 24 and 48 hr posttransfection and used immediately to infect bone marrow cells four times by 90 min spinfection (1000 g). Recipient animals (C57BL/6J, Jackson Labs) were lethally irradiated with 9 Gy total body γ-IR (Gamma Cell cesium source) and transplanted with 2.5 × 10^5 bone marrow cells.

In Situ Apoptosis and Mitosis
Formalin-fixed tissue sections were stained using an in situ cell death kit (Roche) per the manufacturer’s instructions and counterstained with DAPI. Mitotic cells were counted based on morphology, and apoptotic cells were identified by Cy-3 label after TUNEL reaction.

**Histology and Immunohistochemistry**
Tissues harvested from lymphoma-bearing animals were fixed in 4% neutral buffered formalin and then sectioned into 5 μm thick sections for H&E and immunohistochemical stain. Prior to antibody hybridization, antigens were retrieved by boiling slides for 10 min in 10 mM citrate solution. Positive staining was routinely assessed by comparing serial sections exposed to either specific primary antibodies or antibody dilution buffer only (negative controls). All subsequent steps were identical. p16INK4a antibody (F-12 Santa Cruz; 1:100) and p15INK4b antibody (Ab-6 Lab Vision; 1:50) were visualized with a Vectastain Elite kit.
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References


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